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Identification of a Satellite Double-Stranded RNA in the Parasitic Protozoan *Trichomonas vaginalis* Infected with *T. vaginalis* Virus T1

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Co-infection by a 0.5-kb small double-stranded (ds) RNA together with *Trichomonas vaginalis* virus (TVV) genomic 4.6-kb dsRNA is commonly observed in a number of *T. vaginalis* isolates. By molecular cloning and primer extension experiments, the 497-bp cDNA sequence of a 0.5-kb dsRNA co-infecting with TVV-T1 in *T. vaginalis* T1 isolate was elucidated. Consistent with the replication cycle of a typical dsRNA virus, a plus-strand viral RNA beginning at +1 of the 0.5-kb dsRNA was identified in infected *T. vaginalis* T1 cells by primer extension and Northern hybridization studies. The 0.5-kb dsRNA was separately encased in TVV capsids from the viral genomic dsRNA, as shown by protein analysis and electron microscopic examination of viral particles purified by multiple rounds of CsCl gradient centrifugation. The riboprobes transcribed from a cloned cDNA of the 0.5-kb dsRNA exhibited strong hybridization to a small dsRNA in a *T. vaginalis* T9 isolate, which harbors a TVV-T9 distantly related to TVV-T1, but the same probes showed very little hybridization to the viral genomic dsRNA of both TVV-T1 and TVV-T9. Very little sequence homology between the 0.5-kb dsRNA and the 4.6-kb dsRNA in TVV-T1 was found by computer-assisted analysis, suggesting that the small dsRNA in *T. vaginalis* T1 is not derived from the genome of TVV-T1 or other distantly related *T. vaginalis* viruses. These results suggest that the small dsRNAs in *T. vaginalis* are satellite RNAs of *T. vaginalis* virus.

INTRODUCTION

Trichomonas vaginalis virus (TVV) infects many strains of the human protozoan pathogen *T. vaginalis*, the organism which parasitizes the human vagina and causes sexually transmitted vaginitis worldwide. The genome of TVV comprises a single segment of double-stranded (ds) RNA encased in an icosahedral capsid which consists of a major ~75-kDa capsid protein (Wang and Wang, 1986; Tai and Ip, 1995). TVV genomic dsRNA has been shown to upregulate the expression of a phenotypically variable immunogen of *T. vaginalis*. Therefore, TVV has been implicated as a potential virulence factor of the organism (Wang *et al.*, 1987; Khoshnan and Alderette, 1994).

The genomic dsRNA of TVV in various *T. vaginalis* isolates is highly divergent and has been categorized into three groups on the basis of RNA–RNA hybridization (Tai *et al.*, 1993). The heterogeneity is also reflected by the existence of multiple genomic dsRNA fragments ranging between 4.5 and 5 kb in size in some *T. vaginalis* isolates (Khoshnan and Alderette, 1993; Tai and Ip, 1995). The cDNA sequence of one *T. vaginalis* virus in a *T. vaginalis* T1 isolate is 4647 bp long and encodes a capsid protein and an RNA-dependent RNA polymerase (Tai and Ip, 1995).

A 0.5-kb dsRNA has been identified as accompanying TVV infection in some of the virus-infected *T. vaginalis* isolates (Tai *et al.*, 1993). Although the biological consequences of TVV infection in *T. vaginalis* are still speculative, it is possible that this small dsRNA is also involved in the interactions between the virus and the host cells. In this report, a 0.5-kb dsRNA associated with the TVV in a *T. vaginalis* T1 isolate was cloned and sequenced. This small dsRNA is a satellite viral RNA of TVV-T1 because it has little sequence similarity to the 4.6-kb dsRNA of TVV-T1 and yet it is packaged into TVV capsids separately from the 4.6-kb dsRNA.

MATERIALS AND METHODS

Cultures and virus purification

T. vaginalis axenic cultures were maintained as described (Wang and Wang, 1986). *T. vaginalis* isolates T1, T9, and NIH-C1 are infected by TVV, and *T. vaginalis* RU382 is an isolate without detectable TVV (Tai *et al.*, 1993). Viruses were purified from the cell lysates of *T. vaginalis* as previously described but with modifications

The nucleotide sequence data reported in this article have been deposited with the GenBank Database under Accession No. U15991.

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(Tai *et al.*, 1993). Briefly, cells were recovered from ice-cooled cultures by centrifugation at 1500 *g* for 20 min (Beckman, JA10). Protease inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone was added to the cells to a final concentration of 250 μg ml^{-1}. Cell lysates were prepared by sonication, and the debris was removed by centrifugation at 10,400 *g* for 10 min. The viruses were precipitated by 7% polyethylene glycol (MW 6000) and recovered by centrifugation at 6370 *g* for 10 min (Beckman, JA10). The pellets were resuspended in 1% Triton X-100 and 1 *M* NaCl in phosphate buffer, and viral particles were then sedimented by centrifugation at 264,000 *g* for 30 min (Beckman, TLA 100.3). The pellets were resuspended in phosphate-buffered saline (PBS) and adjusted to 1.35 g ml^{-1} by CsCl for gradient centrifugation at 257,000 *g* (Beckman, SW60) for 20–24 hr. Fractions were aliquoted and dialyzed in PBS containing 10% glycerol. In some experiments viruses were washed in detergent and high-salt buffer as described above for subsequent CsCl gradient centrifugation to remove cellular contaminants.

RNA preparation and gel electrophoreses

RNA was extracted from *T. vaginalis* trophozoites or from purified TVV by the hot phenol method as described elsewhere (Scherrer and Darnell, 1962). Agarose gel electrophoresis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described (Sambrook *et al.*, 1989).

Viral cDNA construction

Viral cDNA was constructed as previously described (Tai *et al.*, 1993), but with some modifications. Briefly, the 0.5-kb dsRNA was obtained from viral particles purified by CsCl density gradient centrifugation. The RNA was tailed with a poly(A) at the 3′-terminus by poly(A) polymerase and denatured in 90% DMSO by heating at 80° for 10 min. The cDNA was synthesized by priming the RNA with oligo(dT) and random hexamers simultaneously using the Riboclone system following the supplier's instructions (Promega), and was then cloned into the *Sma*I site of the vector pBluescript (Stratagene) by blunt-end ligation. The recombinant plasmids were then transformed into Sure strain of *Escherichia coli* competent cells (Stratagene). Positive clones were identified by probing the colony lifts with ^{32}P-labeled first-strand cDNA synthesized from the 0.5-kb dsRNA. The viral origins of the inserts in positive clones were further verified by Northern blotting using these inserts as probes.

DNA sequence analysis

The nucleotide sequences were determined on both strands of the cDNA by the dideoxy chain termination method as described by the supplier (United State Biochemical Corp.). Sequence analysis was conducted using the GCG (Genetics Computer Group) program from the University of Wisconsin (Devereux *et al.*, 1984).

Primer extension and RNA sequencing

Primer extension and RNA sequencing were performed as described with some modifications (Geliebter *et al.*, 1986). Briefly, 20 μg cellular RNA and ^{32}P-labeled oligonucleotide p102 (^{436}TTGCCGATGTCAATCTTC-ATCT^{457}) or p103 (^{106}GAATACATTACCGCCTATCCCG^{85}) were mixed in 12 μl annealing buffer containing 0.5 *M* KCl, 20 m*M* Tris–HCl, pH 8.3, and 2 m*M* EDTA. The mixture was heated to 80° for 3 min and incubated at 59° for 1 hr. When the 0.5-kb dsRNA was used as template in this reaction, 5 ng dsRNA was denatured in 90% DMSO at 69° for 10 min before it was added to the annealing buffer. To 2 μl of the annealing mixture, 3.3 μl of transcription buffer containing 24 m*M* Tris–HCl, 3.2 m*M* $MgCl_2$, 8 m*M* dithiothreitol, 0.4 m*M* each of dATP, dTTP, and dCTP, and 0.8 m*M* dGTP, 100 μg ml^{-1} actinomycin D, and 7.5 units AMV reverse transcriptase (Promega) were added. The mixture was incubated at 50° for 1 hr. The extension products were then examined by sequencing gel.

For dideoxy chain termination RNA sequencing, in each reaction one of the following dideoxynucleotides, 0.15 m*M* ddCTP, 0.15 m*M* ddGTP, 0.15 m*M* ddATP, and 0.3 m*M* ddTTP, was added to the annealing mixture as described above. The RNA in the extension mixture was digested by 10 μg RNase A at 37° for 30 min and purified by DNA extraction and ethanol precipitation before loading onto the sequencing gel. The ambiguous reading of the sequencing reaction was resolved by shift-base reaction as described earlier (Deborde *et al.*, 1986).

^{32}P-labeling of the probe and nucleic acid hybridizations

DNA probes were prepared from a combination of cDNA clones C118 (see Results) and p123 (derived from the 4.6-kb dsRNA of TVV-T1; Tai and Ip, 1995) by a Klenow enzyme reaction (Sambrook *et al.*, 1989). Strand-specific ^{32}P-labeled riboprobe was transcribed from viral cDNA clone C118 by T7 RNA polymerase or T3 RNA polymerase as suggested by the supplier (Stratagene) and named accordingly as T7 riboprobe or T3 riboprobe. The T3 riboprobe was complementary to minus-strand RNA and should hybridize only to the dsRNA. The T7 riboprobe was complementary to the plus-strand RNA and should hybridize to both the dsRNA and plus-strand RNA. The RNA was fractionated in a 1% agarose gel and denatured *in situ* for subsequent blotting as described (Furfine *et al.*, 1989). The blot was prehybridized in a hybridization buffer containing 50% formamide without probe for 2 hr at 55°, and the hybridizations were then performed at the same temperature overnight. Subse-

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  1  CTTAAAGAAG CGATAGGAAG TCTTTAAGTG TATTCGGTGC AAACCGTATA
 51  CGAGTGTCCC GGCACCTACT ATCTGACGAA GCCCCGGGAT AGGCGGTAAT
101  GTATTCTTTT GTGAGGAAGA ATAAGCCTTC ACAGGCCAGC CTATGCTGAG
151  CGCGTCAGTG GTAGTGGTTC GTACAGCGCC CTTTCTGGTC GGCTCACATC
201  GAGCAGATTG CGTATGAGAA TGCTATACCA GCCCTCAGAT GCAGTCATAT
251  TACGGACGTG TGGCTACTTT AGTCCTAGGT AAGTAGTGTT CGTAGTATGT
301  GTTATAGGGA GACTTGAAGT CCTAGCAAGC CTACCTTATG GCACGCAACT
351  GTGTCTGTCG TATTACTGGG ATAATGCATA TGTCTGTGCG TCTCGGAGAT
401  ACTCATGCGA TGTCATATGA GATTGACCAA CCGAATTGCC GATGTCAATC
451  TTCATCTGGC GTTTGCACTG AATATCTGCG GCCTCCTATT GACAGTC
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FIG. 1. The cDNA sequence of the plus-strand RNA of the TVV-T1 0.5-kb dsRNA.

quent washings were done in 2× saline sodium citrate with 0.5% SDS at room temperature for 30 min twice and in a solution containing 0.1× saline sodium citrate and 0.1% SDS at 65° for 30 min twice (Sambrook *et al.*, 1989). The hybridization signal was then detected by autoradiography.

Electron microscopy

Viral samples were adsorbed onto Formvar-coated nickel grids and fixed by 0.1% glutaraldehyde. The samples were stained by 0.5% uranyl acetate for subsequent examination by a transmission electron microscope (Jeol, JEM-1200 EX).

RESULTS

The cDNA sequence of the 0.5-kb dsRNA

A 497-nt cDNA sequence of the 0.5-kb dsRNA in *T. vaginalis* T1 (Fig. 1) was obtained from the results described below. First, several cDNA clones from one terminus as indicated by a 3′-poly(A) tailing (e.g., clone C43, nt 239–497) or from internal regions of the 0.5-kb dsRNA (e.g., clone C14, nt 62–377) were obtained by cDNA cloning. These clones were all contained in a cDNA clone C118 (nt 23–497), in which the insert size was 475 bp.

Primer extension was then performed to identify the plus strand of the 0.5-kb dsRNA and to map the start site of this plus-strand RNA. Oligonucleotides p102 and p103, which primed different strands of the 0.5-kb dsRNA, were used. Priming cellular RNA from *T. vaginalis* T1 isolate with p103 resulted in a major extension product of 106 nt which was 22 nt beyond the cDNA sequence of C118 (Fig. 2A). Minor extension products of larger sizes were also seen and are probably due to the existence of a 1-kb dsRNA cross-hybridizing with the 0.5-kb dsRNA (see below). No extension product was obtained when the cellular RNA was extended by p102 (data not shown). The denatured 0.5-kb dsRNA extended by p103 also generated a 106-nt extension product (Fig. 2A). These results suggest that the plus strand of the 0.5-kb dsRNA is present in the TVV-infected *T. vaginalis* T1 and that it starts at +1 of the 0.5-kb dsRNA. The 5′-terminus of the plus strand of the 0.5-kb dsRNA was determined by dideoxy chain termination of the primer extension reactions (Fig. 2B). As expected, reactions in all four lanes terminated at the same position, which masked the reading of the last nucleotide. The ambiguity was resolved by a shift-base reaction of terminal deoxynucleotidyl transferase (Fig. 2C), which clearly indicated that the last nucleotide is G. A total of 51 nt complementary to the template were read from this sequencing, which resulted in the resolution of 22 nt beyond C118. This result is in good agreement with that obtained from primer extension. The sequence of the 3′-terminus was verified by RT–PCR cloning (data not shown). All clones obtained by RT–PCR were identical, and the sequence was also identical to the 3′-terminus of C118 except that no poly(A) stretch was seen in PCR clones.

Numerous termination codons for protein translation were located in all six reading frames on both strands of the 0.5-kb dsRNA, suggesting that the 0.5-kb dsRNA does not contain coding information. The secondary structure of the plus strand of the 0.5-kb dsRNA was predicted by the method of Zucker and Stiegler (1981) using the GCG program (Devereux *et al.*, 1984). Folding of the whole molecule showed extensive intramolecular base-pairing, and the 5′-end of the molecule was complementary to the 3′-end, forming a panhandle-like hairpin structure (Fig. 3A), which is similar to that in a number of satellite RNAs and a defective interfering (DI) dsRNA of reovirus (Roossinck *et al.*, 1992; Zou and Brown, 1992). Folding of the 5′-end, the presumed recognition site for RNA transcription in dsRNA viruses (Wickner, 1993), produced a stem–loop between nt 1 and 28 (Fig. 3B). Folding of the 3′-end produced another stem–loop between nt 476 and 495 (Fig. 3B), which is similar to the replication signal of the plus strand of M1 or X dsRNA of *Saccharomyces cerevisiae* virus (Esteban *et al.*, 1989), except that the plus strand of the 0.5-kb dsRNA contained an adenosine residue protruding from the stem. A similar structure was predicted near the 3′-end of the plus strand of the TVV 4.6-kb dsRNA (Fig. 3C), but with a bulging uridine residue in the stem. The significance of these putative structures in TVV cannot be evaluated at present due to the lack of *in vivo* or *in vitro* assays for viral RNA replication in TVV.

Identification of the capsid protein encasing the 0.5-kb dsRNA

TVV-T1 was purified from 500 ml *T. vaginalis* T1 culture by CsCl gradient centrifugation. The RNA of each fraction in the gradient was analyzed by agarose gel electrophoresis (Fig. 4A). The 4.6-kb viral dsRNA was found in fractions 8 to 13. The 0.5-kb dsRNA was barely visible in this gel. The viral RNA was then examined by Northern hybridization (Fig. 4B) by a combination of DNA probes

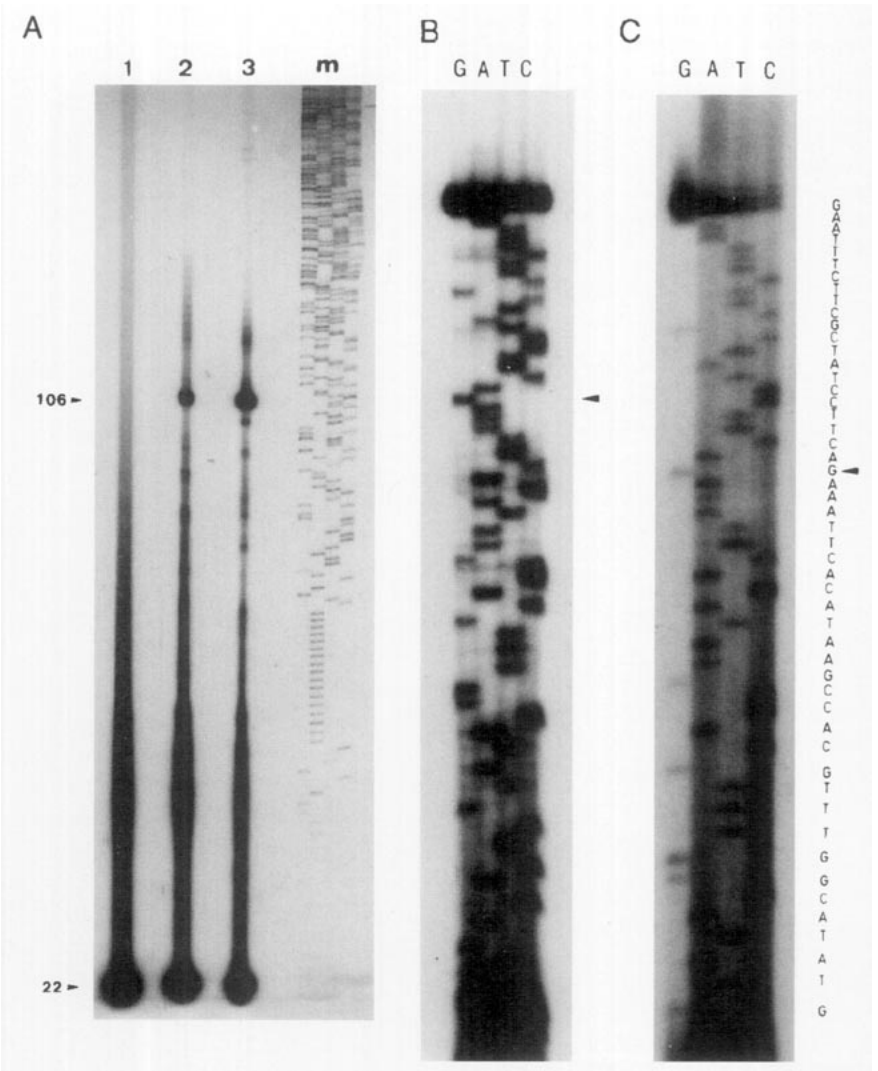

FIG. 2. Primer extension and RNA sequencing of the TVV-T1-associated 0.5-kb dsRNA. (A) Five nanograms denatured dsRNA (lane 2) or 20 μg cellular RNA (lane 3) was mixed with 5′-end ^{32}P-labeled p103 (lane 1) in primer extension reactions. (B) Dideoxy termination of the extension product was performed. (C) The extension products from B were further extended by terminal deoxynucleotidyl transferase to resolve the ambiguity of the last nucleotide. Arrows indicate the terminus of cDNA C118 sequence.

derived from C118 and cDNA clone p123, which was derived from the TVV-T1 4.6-kb dsRNA (Tai and Ip, 1995). Strong hybridization to the 0.5-kb dsRNA was found in fractions 2 to 11 of lighter CsCl buoyant density, and two peaks were found at fractions 3 and 6. A faint 1-kb band was seen in fractions 2, 3, 6, and 7. The 4.6-kb dsRNA was found in the more dense fractions 6 to 15, and a peak was found in fraction 10. Both RNA species were found in fractions 6 to 11. Both RNA species were also precipitated to the bottom fraction of the CsCl gradient, implying that the RNA molecules in this fraction were not covered with a protein coat.

To examine whether the 0.5-kb dsRNA is encased within the TVV capsid, the distribution of capsid protein and viral RNAs in a CsCl gradient purified from *T. vaginalis* T1 (Fig. 5A) was compared to that of those from *T. vaginalis* NIH-C1 (Fig. 5B), an isolate infected by TVV but without the co-infection of the 0.5-kb dsRNA (Tai *et al.*, 1993). In these experiments TVV was purified from 5 liters of each *T. vaginalis* culture. An ~75-kDa protein (P75) reputed to be the viral capsid protein (Tai and Ip, 1995) was copurified with the 0.5-kb dsRNA (fractions 1–10) and the 4.6-kb dsRNA (fractions 7–13) in TVV-T1 samples. Small quantities of the 4.6-kb dsRNA were present in fractions 3–6. In this gradient the amount of viral capsid protein in each fraction was proportional to the amount of viral RNA (data not shown). In TVV-NIH-C1 samples P75 was copurified with the 4.6-kb dsRNA (fractions 8–11), but not in the fractions devoid of viral RNA. Although viral RNAs were greatly enriched by the first CsCl gradient centrifugation, numerous cellular contaminants were copurified with the viruses. To confirm the

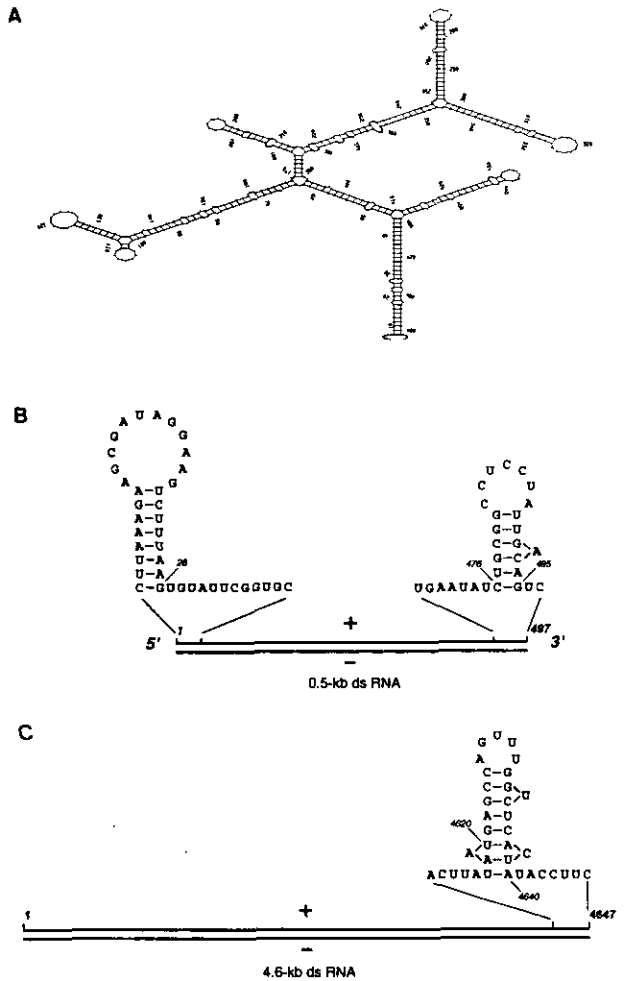

FIG. 3. Secondary structure prediction of the plus strand of the 0.5-kb dsRNA. The method of Zucker and Stiegler (1981) in a computer program, GCG (Devereux *et al.*, 1984), was used to predict the secondary structure of the whole RNA molecule (A) or for the 5′-end or 3′-end of the molecule (B). The prediction for the 3′-end of the plus strand of the 4.6-kb dsRNA in TVV is shown in C.

coexistence of the 0.5-kb dsRNA and P75, viral samples containing the TVV-T1 0.5-kb dsRNA (fractions 1–6 in Fig. 5A) or similar density fractions of TVV-NIH-C1 samples (fractions 1–6 in Fig. 5B) were pooled for subsequent purification. After a second CsCl gradient centrifugation P75 was again copurified with TVV-T1 0.5-kb dsRNA in fractions 1–4 (Fig. 5C), but it was not found in TVV-NIH-C1 samples (Fig. 5D).

Cellular contaminants in enriched TVV-T1 viral samples as described above were further removed by a third CsCl gradient centrifugation. In viral samples containing exclusively the 0.5-kb dsRNA (Fig. 6A, lane 1) or the 4.6-kb dsRNA (Fig. 6A, lane 2), P75 was found to be a major protein (Fig. 6B). Consistent with previous reports (Wang and Wang, 1986; Tai *et al.*, 1993), an ~60-kDa protein was copurified with the P75 from TVV samples (Fig. 6B, lane 2). From viral samples containing the 0.5-kb dsRNA, instead, an ~50-kDa protein was copurified (Fig. 6B, lane 1). The properties of the ~60-kDa protein and the ~50-kDa protein and their relationships with TVV have not been examined. Icosahedral-shaped virus-like particles (VLP) with a diameter of approximately 25 nm were identified by electron microscopic examination of viral samples containing the 0.5-kb dsRNA (Fig. 6C). Smaller nucleocapsid-like particles were also seen in this viral sample, but their identity is not clear. In the 4.6-kb dsRNA sample most VLP were similar to those found in the 0.5-kb dsRNA sample in size and shape, but only a few nucleocapsid-like particles were present (Fig. 6D). A larger icosahedral-shaped particle (~45 nm) was also seen. The distributions of the 0.5-kb dsRNA and the 4.6-kb dsRNA in CsCl gradients and their associations with VLP consisting of similar capsid protein suggest that they are encased within similar capsids, but that they do not coexist in the same individual particles.

Nucleic acid hybridization

The small dsRNAs in two *T. vaginalis* isolates T1 and T9 that harbor two distantly related TVVs (Tai *et al.*, 1993)

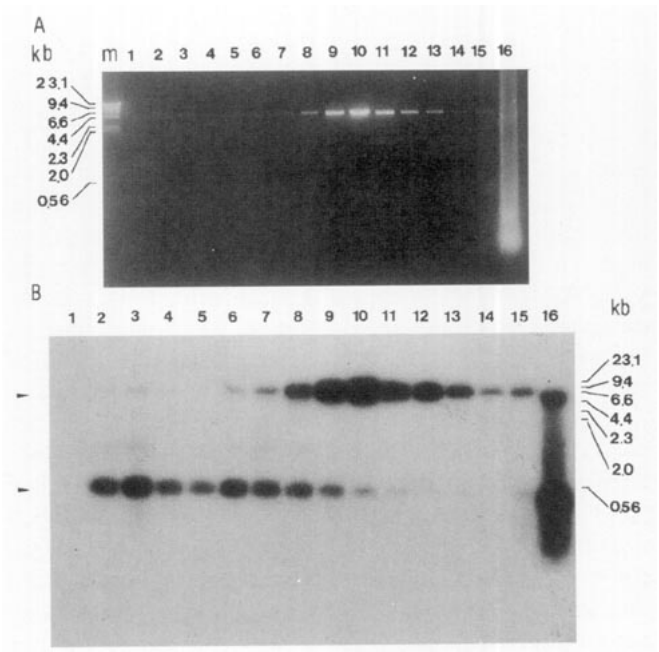

FIG. 4. Distribution of the 0.5-kb dsRNA and the viral genomic 4.6-kb dsRNA in a CsCl gradient. TVV was purified from cell lysate of *T. vaginalis* T1 isolate by CsCl gradient centrifugation. The gradient was divided into 16 fractions from the top of the gradient (lane 1) to the bottom (lane 16). (A) RNA in each fraction was extracted, separated by a 1% agarose gel, and stained with ethidium bromide. (B) The RNA in the gel was denatured, blotted onto Nytran membrane, and hybridized with ^{32}P-labeled viral cDNA clone p123 (derived from the TVV-T1 4.6-kb dsRNA) and C118 (derived from the TVV-T1 0.5-kb dsRNA). λDNA digested with *Hin*dIII was used as size markers.

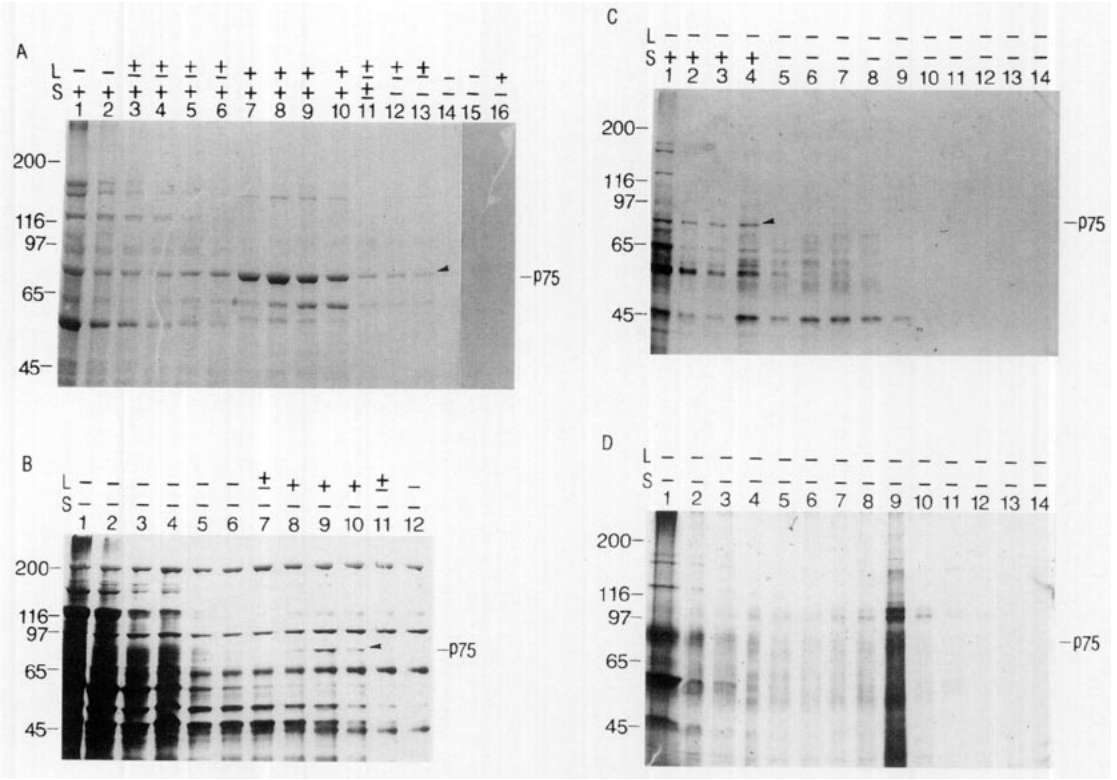

FIG. 5. Coexistence of viral capsid protein and the viral dsRNA. Cell lysates obtained from *T. vaginalis* T1 (A) or *T. vaginalis* NIH-C1 (B) were fractionated by CsCl gradient centrifugations. The gradients were aliquoted from the top of the gradient (lane 1) to the bottom. Viral proteins in each fraction were analyzed in 7.5% gels by SDS–PAGE, and the proteins were detected by silver staining. The presence or absence of the 4.6-kb dsRNA (L) or the 0.5-kb dsRNA (S) in each fraction analyzed by agarose gel electrophoresis is indicated as + or –, respectively; and ± indicates the presence of relatively small quantities of respective viral RNAs. (C and D) Viral samples from fractions 1–6 in A and fractions 1–6 in B, respectively, were pooled for a second CsCl gradient centrifugation. Arrowhead indicates the capsid protein P75.

were analyzed by Northern hybridization. Cellular RNA from *T. vaginalis* isolates T1, T9, or RU382 or dsRNA from purified TVV-T1 was included. RNA samples were fractionated in a 1% agarose gel and stained by ethidium bromide (Fig. 7A). The RNA in the gel was denatured, transferred to Nytran membrane, and probed by ^{32}P-labeled T3 or T7 riboprobe derived from cDNA clone C118. The T3 riboprobe cross-hybridized to a 0.5-kb band in RNA from the *T. vaginalis* T1, T9, or TVV-T1 sample (Fig. 7B). A 1-kb band was also seen in the *T. vaginalis* T1 or T9 sample. Consistent with previous observations, no hybridization band at similar positions was detected in samples from virus-free RU382 isolate (Tai *et al.,* 1993). Similar hybridization patterns were seen by T7 riboprobe except that an additional 0.4-kb band was observed in RNA from *T. vaginalis* T1 or T9 samples (Fig. 7C), suggesting that the 0.4-kb band is the plus-strand RNA of the 0.5-kb dsRNA. These probes exhibited very little cross-hybridization to the 4.6-kb viral genomic dsRNA of TVV-T1 or TVV-T9 viruses or any other TVV genomic dsRNAs in our hands, even after prolonged exposure (data not shown).

DISCUSSION

Virus-associated small RNA molecules encased in the capsids of helper viruses can be roughly divided into two groups, the DI RNAs which are derived from the genomes of the helper viruses (Roux *et al.,* 1991) and the satellite RNAs which exhibit very little sequence homology to the helper viruses (Roossinck *et al.,* 1992). Both groups of these small RNAs rely on their helper viruses for replication, and they may have dramatic effects on the clinical symptoms induced by their helper viruses. Since TVV is a potential virulence factor of *T. vaginalis,* it is important to study the small RNAs that co-infect with TVV in this organism. On the basis of minimal sequence homology and a packaging system similar to that of the TVV-T1 4.6-kb dsRNA, in this report we have identified a satellite 0.5-kb dsRNA in the parasitic protozoan *T. vaginalis* T1 isolate.

The riboprobes transcribed from the cloned cDNA of small dsRNA did not cross-hybridize to distantly related TVV genomic dsRNAs on Northern blots. This is contradictory to our previous findings that the small dsRNA exhibited significant hybridization to the TVV 4.6-kb dsRNA (Tai *et al.,* 1993). This discrepancy is probably due to contamination of the small dsRNA preparations by viral 4.6-kb dsRNA in the previous report. In fact, very little sequence homology between the 0.5-kb dsRNA and the 4.6-kb TVV-T1 dsRNA (Tai and Ip, 1995) can be identi-

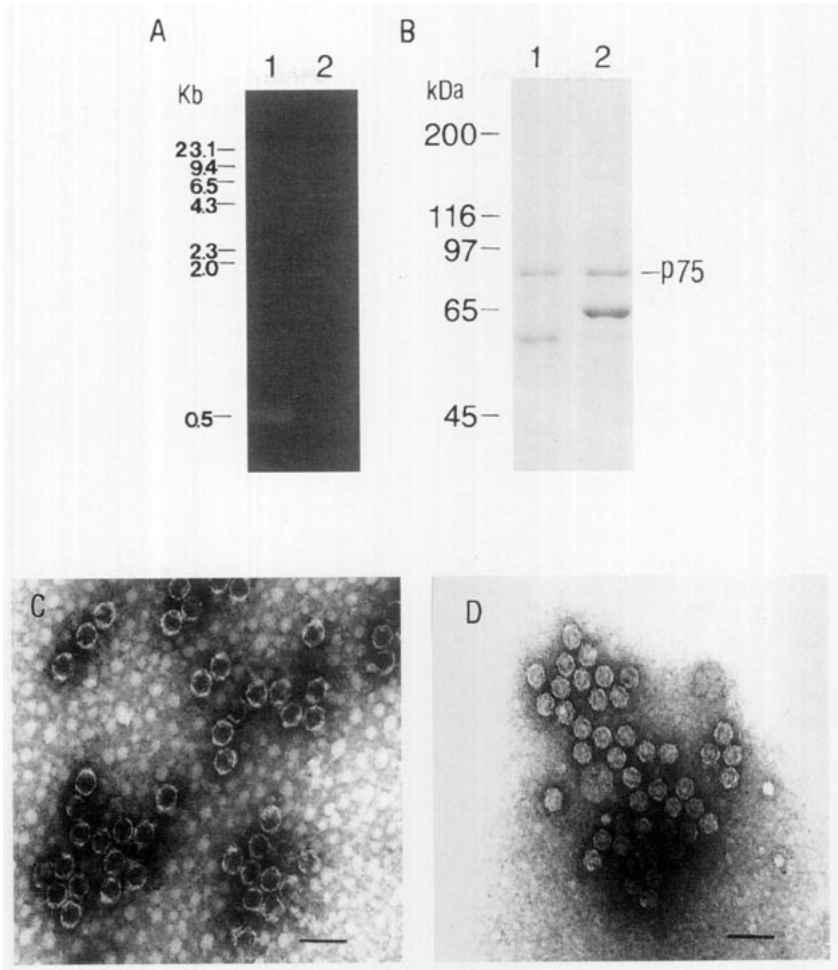

FIG. 6. The capsid protein and electron micrographs of the satellite RNA. Enriched viruses from *T. vaginalis* T1 containing the 0.5-kb dsRNA or the 4.6-kb dsRNA as described in Fig. 5 were pooled and resuspended in 1% Triton X-100 and 1 *M* NaCl in phosphate buffer. Viral particles were sedimented by ultracentrifugation, and the pellets were resuspended in PBS for subsequent CsCl gradient centrifugation. The RNA (A) of a 0.5-kb dsRNA-containing fraction (lane 1) and that of a 4.6-kb dsRNA-containing fraction (lane 2) were analyzed in a 1% gel by agarose gel electrophoresis, and their proteins (B) were analyzed in a 7.5% gel by SDS–PAGE. The RNA gel was stained by ethidium bromide, and the protein gel was stained by Coomassie blue. The viral capsid protein P75 is indicated. VLP containing the 0.5-kb dsRNA (C) or the 4.6-kb dsRNA (D) were stained by 0.5% uranyl acetate for transmission electron microscopic examination. Bar represents 50 nm.

fied by computer analysis. The same riboprobe did not cross-hybridize to the *T. vaginalis* genomic DNA by Southern blotting (unpublished observation). These observations suggest that the satellite dsRNA originates neither from TVV genomic dsRNA nor from the genome of *T. vaginalis*.

A related 1-kb dsRNA in the *T. vaginalis* T1 was also found in very small quantities in viruses purified by CsCl gradient centrifugation, indicating that the 1-kb dsRNA perhaps can also be packaged into viral capsids. The relationship between the 0.5-kb dsRNA and the 1-kb dsRNA is not clear. The 0.5-kb dsRNA in the CsCl gradient was found in a wide range of contiguous density fractions. This is probably due to the presence of multiple 0.5-kb dsRNA molecules within a single capsid, which may form multiple discrete species of VLP with different densities in a CsCl gradient. A similar phenomenon has been reported in the packaging of X dsRNA, a 0.5-kb DI dsRNA derived from L-A dsRNA of *S. cerevisiae* virus (Esteban and Wickner, 1988).

Satellite dsRNAs have been found in many isolates of TVV-infected *T. vaginalis* (Tai *et al.*, 1993). Also, the satellite dsRNAs in different *T. vaginalis* isolates were shown to be more closely related to each other than were their helper viruses, implying a broad range of helper virus specificity for the satellite dsRNAs. The satellite dsRNAs in *T. vaginalis* T1 axenic culture have been maintained through long-term passage, suggesting that they are RNA replicons in *T. vaginalis* T1. The 0.5-kb dsRNA does not encode proteins essential for viral replication, namely the capsid protein and RNA-dependent RNA polymerase (Wickner, 1993), yet it is packaged into the capsids pro-

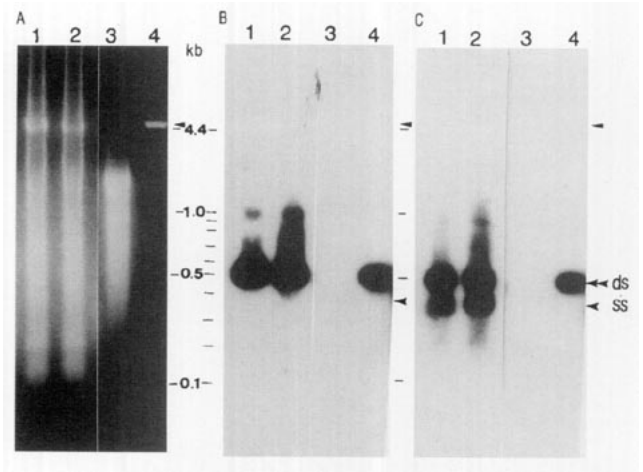

FIG. 7. Northern hybridization of the 0.5-kb dsRNAs in various *T. vaginalis* isolates. RNA from *T. vaginalis* T1 (lane 1), T9 (lane 2), RU382 (lane 3), or purified TVV-T1 (lane 4) was fractionated by agarose gel electrophoresis and stained by ethidium bromide (A). RNA in the gel was then denatured and blotted onto Nytran membrane. The blot was then hybridized with ^{32}P-labeled T3 (B) or T7 (C) riboprobe at 55° overnight in a hybridization solution containing 50% formamide. The blot was then washed under conditions described under Materials and Methods. λ DNA digested with *Hin*dIII and a 0.1-kb DNA ladder (BRL) were used as size markers. The 0.5-kb dsRNA is indicated by double curved arrowheads and the respective ssRNA is indicated by single curved arrowhead. The 4.6-kb viral genomic dsRNA is indicated by a plain arrowhead.

vided by TVV. Accordingly, the 0.5-kb dsRNA is probably exploiting the replication machinery provided by TVV for multiplication. A recent report has shown that similar satellite RNAs can be synthesized *in vitro* by the viral RNA-dependent RNA polymerase from partially purified TVV (Khoshnan *et al.*, 1994). Thus the sequence of the 0.5-kb dsRNA must contain *cis*-acting elements for virus multiplication, such as RNA replication, transcription, and packaging. These signals are probably shared between the 0.5-kb dsRNA and the 4.6-kb dsRNA of TVV-T1. Since sequence homology between these two RNA species is insignificant, they probably recognize similar RNA secondary structures for viral replication or even exploit different strategies to replicate.

The features of the 0.5-kb dsRNA described above fulfill the criteria of a satellite RNA, and information obtained from this study will allow us to examine the interactions between the satellite RNA and TVV in the future.

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